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Previous results (Year 1) indicated that human breast epithelial cells transfected with ErbB2 exhibited increased activity, but not expression, of c-src. This increase was associated with decreased phosphorylation on tyrosine 527. In year 2, the expression and activity of C-terminal src kinase (CSK), responsible for phosphorylating src on Y527 was found to be increased by ErbB2. However, an even greater increase in activity of phosphatases directed toward the C-terminus of src was observed. This phosphatase was found to physically associate with ErbB2, and has been tentatively identified as SHP2. This suggests that ErbB2 physically associates with and activates SHP2, which removes an inhibitory phosphate at position 527 in src. Preliminary results using inhibitor src constructs indicate that src activation by ErbB2 may be important in mediating some aspects of tumor progression. In particular, the ability of cells to grow on soft agar was inhibited by inhibition of src activation. These results suggest that src, or the kinases and phosphatases regulating src, may provide useful targets for breast cancer therapy.

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Susan B. Wright 7/29/98

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INTRODUCTION

The hypothesis of this proposal is that ErbB2 expression regulates the activity of c-src and that this activation is a factor in mediating ErbB2 induced tumorigenesis. Previous work with human tumors has shown increased c-src activity (Ottenhoff-Klaff et al., 1992). Tissue from mice overexpressing ErbB2 show increased activity of c-src (Muthuswamy et al., 1994). Knockout mice lacking c-src also exhibit decreased tumorigenesis induced by polyomavirus middle T antigen (Guy et al., 1994). Data from our laboratory and others (Zhair et al., 1993) show that non-tumorigenic mammary epithelial cells are readily transformed to a tumorigenic phenotype by overexpression of ErbB2. This tumorigenic transformation is accompanied by increased activity of c-src (see below).

The overall project has two major areas of focus: the mechanism by which ErbB2 activates c-src and the consequences of c-src activation. The first year's work focused on the activation of c-src by ErbB2. There are several ways by which ErbB2 could regulate c-src activity: by altering c-src expression levels, by decreasing expression and/or activity of csk, by increasing expression and/or activity of c-src-directed phosphotyrosine phosphatases or by phosphorylation-independent activation of c-src. These possible mechanisms were addressed in Year 2 work.

As described in the STATEMENT OF WORK, year 2 consisted of assessing effects of ErbB2 on c-src kinases and phosphatases. This consists of:

A. Determine the effect of ErbB2 on cellular content and activity of src kinases (CSK)

B. Determine the effect of ErbB2 on activity and content of Src Phosphatases

C. Begin the process of identifying the specific phosphatases activated by ErbB2

In addition, Year 2 also involves generation of various cell lines that will be used for investigations in years 3 and 4.

BODY

Experimental Methods

Cell Lines

Parental Lines

The nontumorigenic human mammary epithelial cell line 184.A1 and the nontumorigenic mouse line NMuMG was used in most studies covered by this report. Cells were routinely grown in DMEM:F12 + 10% FBS, 5 μg/ml insulin and 10 ng/ml EGF.

ErbB2 and Ras transformed lines

Cells were transfected by ErbB2 and with v-Ha-ras in vectors containing a G418 resistance marker. Vectors were cloned in DH5α E. coli using standard procedures. Cells ($5x10^6$ in 0.5 ml HBSS) were placed in electroporation cuvets with 2 mm electrode space and pulsed with 1.2 kV/cm field strength. Cells were left on ice for 10 minutes, then returned to culture media. After 24 hours, media was changed to contain 400 μg/ml G418 sulfate and selection continued for 4 weeks.

ErbB2 and dominant negative src transfected lines

For studies on the functional consequences of c-src activation by ErbB2, cells were initially transfected with dominant negative c-src under control of the CMV promoter and in a neomycin resistance vector (Bell et al., 1992) or with empty vector. These cells were then selected for stable transfection by selection in media containing G418 to give control and dominant negative c-src transformed cells.

The ErbB2 was then excised from the initial neomycin resistance vector and subcloned into a Zeocin resistance vector. Control and dominant negative c-src cells (above) were then transfected with this vector or with the empty vector and selected in media containing G418 and Zeocin.

Growth on soft agar

Cells (10^4) were suspended in 2 ml of 0.3% agarose dissolved in culture media and layered onto 2 ml of hardened 0.5% agarose in culture media. After 10 days, cells were observed for growth.

Measurement of CSK content

Cells were lysed with SDS loading buffer and proteins separated by SDS-PAGE. Proteins were transferred to PVDF membranes, blocked with BSA and probed for CSK by Western blot analysis using chemiluminescence detection. Computer assisted densitometry was used to estimate relative band intensity.

Measurement of CSK activity

Cells were lysed with lysis buffer (50 mM HEPES containing 1 mM PMSF, 40 mM Sodium orthophosphate, 1 mg/ml BSA and 1% Triton X-100), clarified by centrifugation (14,000 g for 15 minutes) and CAK immunoprecipitated from lysates using a rabbit polyclonal antibody and agarose conjugated protein A/G. Beads were washed with lysis buffer and then incubated with assay buffer (200 mM HEPES containing 100 mM $MgCl_2$, 25 mM $MnCl_2$ and 0.1 mM Na_3VO_4) with substrate peptide corresponding to the C-terminus of src (TSTEPQY(PO_4)QPENL). Assays were begun by adding γ^{32}P-ATP (10 μM containing 250,000 dpm) and continued for 5 minutes. Assays were stopped by adding 50% acetic acid and 1 mg/ml BSA. Tubes were then centrifuged (14,000 g for 5 minutes) and supernatant spotted onto Whatman 3MM filter Paper. Filter paper was washed four times with 100 mM phosphoric acid, dried and counted by liquid scintillation. Background was estimated by performing the assay in the absence of substrate peptide and was subtracted to give substrate-dependent activity.

Measurement of phosphatase activity

For assays on crude cell lysates, cells were lysed with 50 mM HEPES containing 40 mM sodium pyrophosphate, 50 mM NaF, 0.1 mM Na^3VO^4 , 1 mM PMSF and 1% Triton X-100. Lysate was concentrated with an Amicon centrifical concentrator and resuspended in phosphate-

free buffer. Protein content was determined by BCA method (Pierce Chemical Co., Rockford, IL), equalized among samples and phosphatase activity assessed by the Malacite Green method of assessing free phosphate (Harder et al., 1994) and using a peptide corresponding to the C-terminus of src (peptide 301, Biomole, Plymouth Meeting, PA) or a peptide corresponding to the autophosphorylation site of src (peptide 312, Biomole) as substrate peptides.

For membrane preparations, cells were lysed as described above except that the buffer lacked Triton X-100, centrifuged (2000 g for 5 minutes) and supernatant centrifuged at 50,000 g for 60 minutes. Pellets were dissolved in phosphate free assay buffer containing 1% Triton X-100 and phosphatase activity assessed as described above. Cytosol was assessed as described above for cell lysates.

To assess phosphatase associated with ErbB2, ErbB2 was immunoprecipitated and resulting immunoprecipitates assayed as described above.

Western Analysis of phosphatase activity

Cell lysates, membranes or ErbB2 immunoprecipitates were prepared as described above. Proteins were separated by SDS-PAGE, transferred to PVDF membranes and western blots probed with antibodies to the indicated phosphatases. Computer densitometry was used to assess relative band intensity.

Results

Summary of Year 1 Results

For background and to place Year 2 results in context, Year 1 results are summarized briefly. Data supporting these conclusions were reported in year 1 Progress Report.

1. ErbB2 vector efficiently increased expression of ErbB2 in mammary epithelial cells
2. ErbB2 transfection leads to a transformed phenotype, as assessed by growth on soft agar and in athymic mice
3. ErbB2 transformation leads to increased activity of c-src without increasing expression of c-src.
4. The c-src assay was linear over time and amount of enzyme, was dependent on added substrate and conducted under substrate and ATP concentrations leading to maximum activity. Results did not appear to be due to suboptimum assay conditions.
5. C-src phosphorylation pattern in ErbB2 transformed cells was consistent with dephosphorylation at Y527 in response to ErbB2.
6. These results appeared to be reproducible in other mammary epithelial cell lines.

Expression and Activity of CSK

Since a possible mechanism by which ErbB2 could increase c-src activity is to decrease expression or activity of C-terminal Src Kinase (CSK), we examined the expression and activity of CSK in ErbB2 and Ras transformed cell lines. Ras had no significant effect on CSK level, whereas ErbB2 significantly increased CSK expression level (Figure 1). These effects were paralleled by a similar increase in CSK activity. When corrected for differences in CSK content, there was no effect of ErbB2 on specific activity of CSK. Thus, it appears that ErbB2 does not activate c-src by decreasing the rate at which Y527 is phosphorylated.

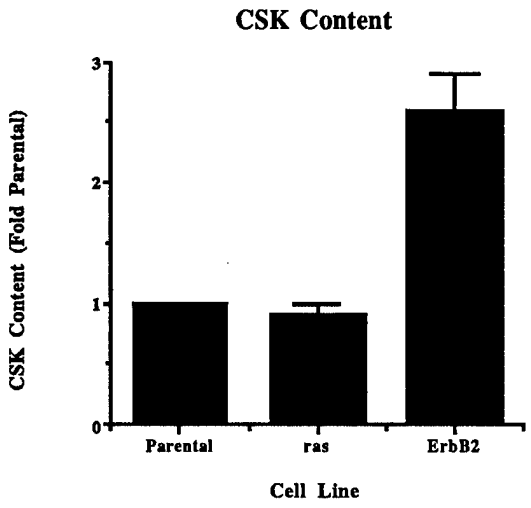

Figure 1. Effect of ErbB2 on CSK expression in 184.1 cells transformed with ErbB2 or ras.

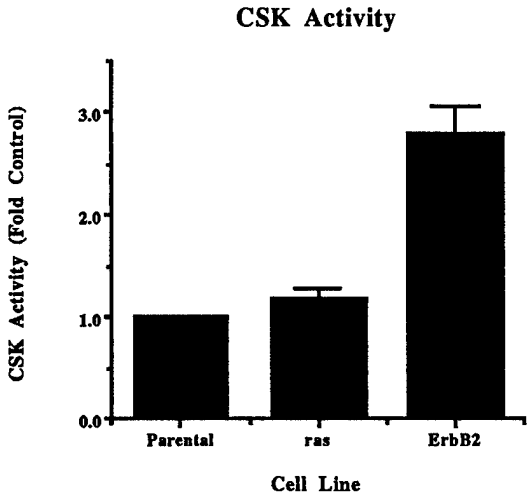

Figure 2. Activity of CSK in 184.1 cells transformed with ErbB2 or ras.

Total Phosphatase Activity

To assess total phosphatase activity in various cell lines, two substrate peptides were used: 312, corresponding to Y416, the autophosphorylation site of c-src, and 301, corresponding to Y527, the CSK phosphorylated site of c-src. Substantially higher activity toward peptide 301 was observed, but there was no difference among cell lines in this activity. Lower activity was observed toward peptide 312, but this activity was slightly higher in ErbB2 transformed cell lines than in other lines.

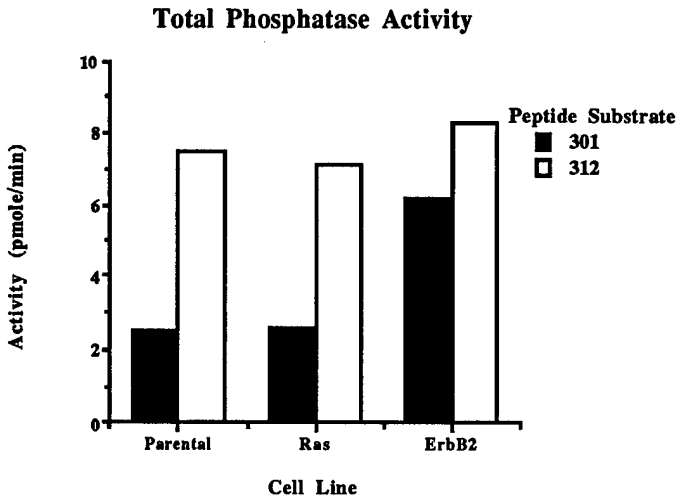

Figure 3. Effect of ErbB2 and ras transformation on total phosphatase activity of 184.1 cells toward c-src autophosphorylation site and CSK phosphorylation site.

Membrane associated Phosphatase Activity

Cell membranes and cytosol prepared from control, ras or ErbB2 transformed cells were assessed for phosphatase activity toward peptide 301 and 312. There was no difference among cell lines in peptide 312 dephosphorylation in either membrane or cytosolic fractions (Figure 5). In contrast, the membrane fraction of ErbB2 transformed cells exhibited substantially greater activity toward peptide 301 than other cell lines (Figure 4). However, the cytosolic fraction showed little difference in activity among the cell lines. These results indicated an increased membrane associated phosphatase activity with activity selective for Y527 of c-src. Further examination of the activity indicated that it was linear over the amount of membrane protein used (Figure 6) and over time (Figure 7). In addition, the activity was dependent on substrate, and near Vmax conditions were used in the standard assay (Figure 8).

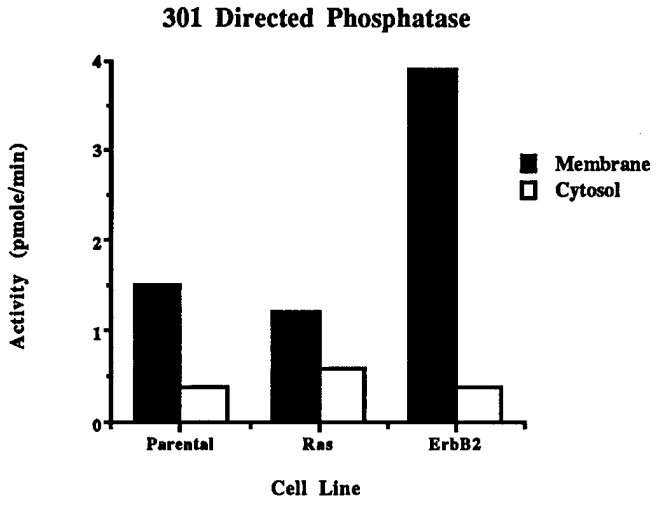

Figure 4. Effect of ErbB2 and ras transformation on activity of phosphatase toward CSK phosphorylation site in cell membranes and cytosolic fractions.

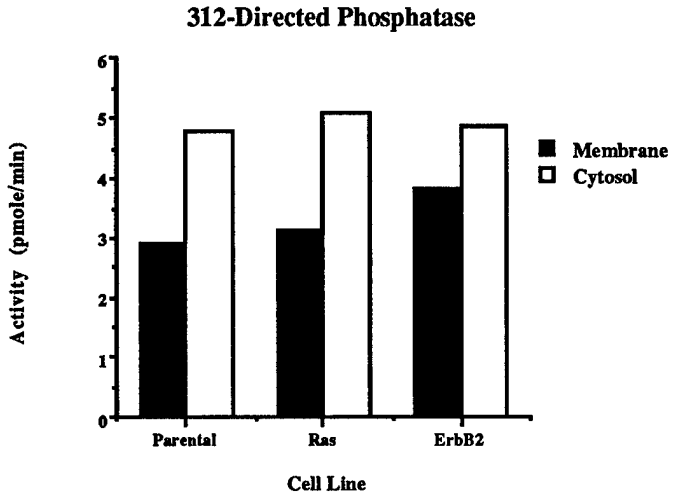

Figure 5. Effect of ErbB2 and ras transformation on activity of phosphatase toward c-src autophosphorylation site in cell membranes and cytosolic fractions.

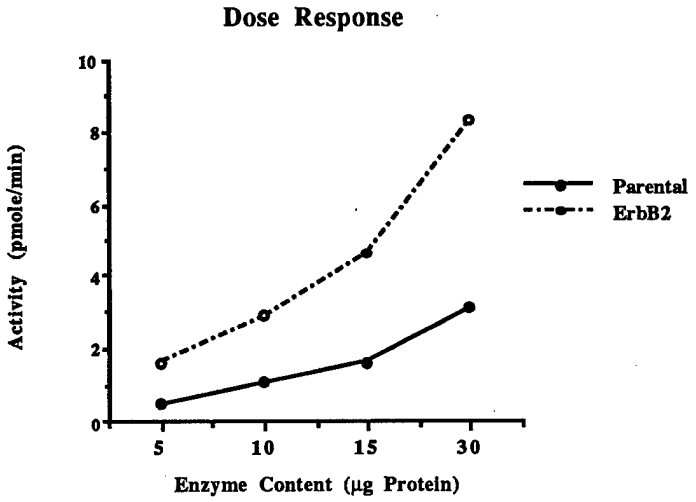

Figure 6. Linearity of phosphatase activity toward peptide 301 (CSK phoshorylation site of src) over amount of membrane protein assayed in Parental and ErbB2 transformed human mammary epithelial cells.

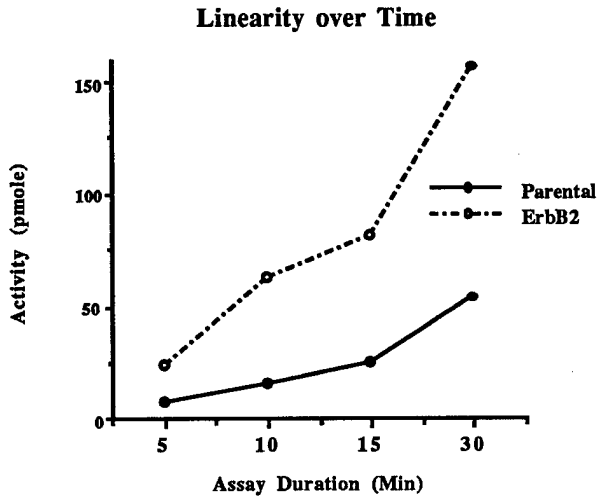

Figure 7. Linearity of phosphatase activity toward peptide 301 (CSK phoshorylation site of src) over time in Parental and ErbB2 transformed human mammary epithelial cells.

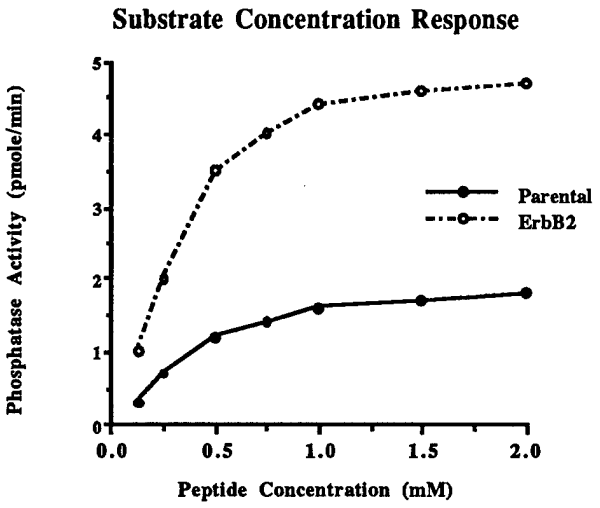

Figure 8. Substrate kinetics of phosphatase activity toward peptide 301 (CSK phoshorylation site of src) over amount of membrane protein assayed in Parental and ErbB2 transformed human mammary epithelial cells.

ErbB2 associated phosphatase activity

Phosphatase activity in ErbB2 immunoprecipitates was assessed essentially as for membrane fractions. These studies indicated that ErbB2 was physically associated with a phosphatase with selectivity toward Y527 of CSK, and that the amount of this enzyme that co-precipitated with ErbB2 was dramatically increased (about 9 fold) in cells transfected with ErbB2 (Figure 9). However, it should be noted that ErbB2 transformed cells express 9-10 fold more ErbB2, so that the increased ErbB2 association may be due to the increased amount of ErbB2, not a specific increase in phosphatase-ErbB2 association in transformed cells.

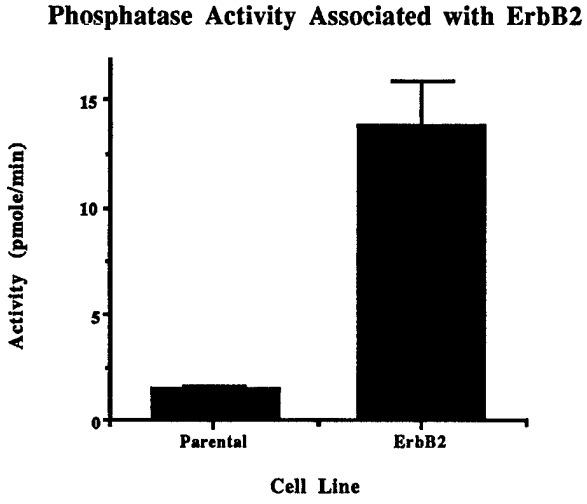

Figure 9. Phosphatase activity toward the CSK phosphorylation site of src in ErbB2 immunoprecipitates prepared from parental and ErbB2 transformed human mammary epithelium.

Specific Phosphatases

Western blot analysis indicated that the cell lines examined expressed detectable levels of PTP1B and LAR, and these were increased by transformation. The cells expressed little SHP1 and SHP1 content was not affected by transformation. The cells expressed substantial amounts of SHP2, and total SHP2 content was actually slightly lower in transformed cells (Figure 10). However, SHP2 associated with membrane fraction was substantially increased in ErbB2 transformed cells. In addition, the amount of SHP2 present in ErbB2 immunoprecipitates was increased about 10 fold, which is similar to the increase in ErbB2. These results, together with previous studies, suggest that SHP2 associates with ErbB2. Transformation with ErbB2 does not dramatically alter the specific association, but, due to greater ErbB2 expression, the total amount of SHP2 associated with ErbB2 is dramatically increased. This provides a plausible mechanism for ErbB2 activation of c-src, although that hypothesis has not yet been definitively tested.

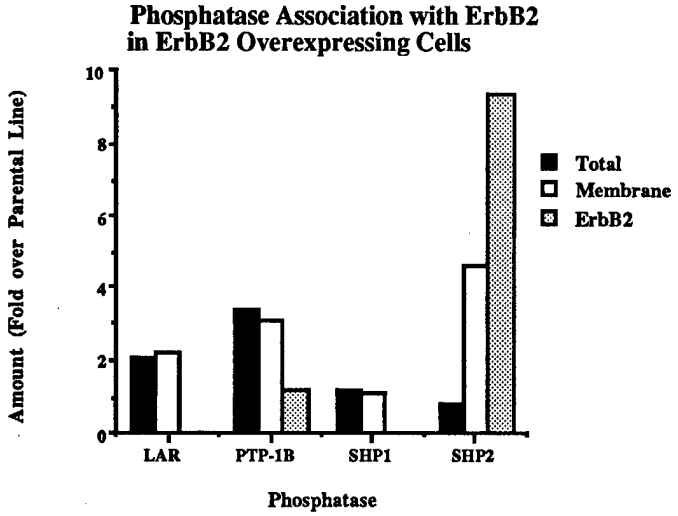

Figure 10. Expression level of specific phosphatases in ErbB2 transformed cell lines. Phosphatase content of crude cell lysates (Total), membrane preparations or ErbB2 immunoprecipitates were determined by Western analysis of parental and ErbB2 transformed human mammary epithelial cells and expressed as ErbB2 expression/Parental expression.

ErbB2-Dominant Negative c-src cell lines

In preliminary studies to characterize cell lines transfected with dominant negative c-src and ErbB2, the dominant negative mutant of c-src did not appear to inhibit cell proliferation on plastic. However, colony forming efficiency on soft agar was dramatically inhibited by dominant negative c-src when cells were transformed by ErbB2, but not by ras (Figure 11). Further evaluations of these cells are necessary, and scheduled for Year 3 of the project. These evaluations include replication of cell growth results, tumor formation in vitro, cell invasiveness assays and evaluations of c-src activity. However, the results to date indicate that c-src, although not necessary for cell proliferation, appears to be critical for ErbB2-mediated tumorigenic transformation.

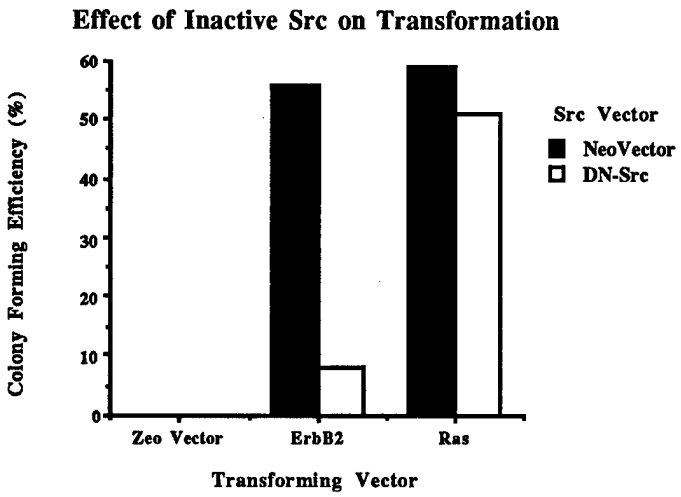

Figure 11. Colony forming efficiency of cells expressing combinations of ErbB2 and dominant negative src. Cells were transfected with a dominant negative kinase inactive src (DN Src) or vector alone (Neo Vector) and selected for G418 resistance. These cells were transfected with ErbB2, ras or vector alone (Zeo vector) and selected for Zeocin resistance. Cells were plated onto soft agar and colony forming efficiency deterined.

Discussion

Previous results (detailed in Year 1 progress report) indicated that c-src was activated by ErbB2 without increasing c-src expression. Standard assay validations indicated that this was due to a true increase in src activity. A major objective of the overall project is to determine how ErbB2 activates c-src. This activation is associated with decreased phosphorylation at Y527. Presumably, the decreased Y527 phosphorylation could be due to decreased CSK activity or increased phosphatase activity. The present studies indicate that decreased CSK activity does not appear to explain src activation by ErbB2. However, increased activity of SHP2 appears to be a plausible mechanism by which ErbB2 could activate src. In the present study, we determined that SHP2 was associated with ErbB2. This association does not appear to be modified by tumorigenic transformation, as the increase in SHP2 associated with ErbB2 immunoprecipitates was largely parallel with increased ErbB2 content of cells.

A second major objective of the project is to determine the consequences of c-src activation by ErbB2. In Year 2, we developed cell lines to be used in assessing this objective. Preliminary results indicate that a major indicator of tumor phenotype, growth on soft agar, was decreased by a dominant negative inhibitor of c-src.

Recommendations

Based on the results of Year 2, the plan of work outlined for year 3 remains plausible. This includes further definition of the specific phosphatases activated by ErbB2 and further assessment of the characteristics of cells transfected with ErbB2 and dominant negative c-src. Current results suggest that the SHP2 phosphatase may be a critical factor in ErbB2 mediated c-src activation. Availability of inhibitory mutants of SHP1 and SHP2 (Servidei et al., 1998) make investigation of this hypothesis by co-transfecting ErbB2 and inhibitory SHP mutants, followed by assessment of tumorigenic phenotype and c-src activity reasonable.

CONCLUSIONS

Previous results indicate that ErbB2 increases activity of c-src in mammary epithelium. The present studies suggest that this induction of c-src activity is mediated by increased activity of a src-directed kinase that removes an inhibitory phosphate at Y527. SHP2 appears to be a leading candidate for the phosphatase. In addition, the activation of c-src by ErbB2 appears to play a critical role in inducing a tumor phenotype. These results that strategies to modify src activity or the activity of the src-directed phosphatase(s) may prove useful in modifying tumor progression.

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APPENDICES

None